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Caring males do not respond to cues about losses in paternity in the burying beetle *Nicrophorus vespilloides*

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In species with biparental care, males may be under selection to adjust the amount of care they provide for their offspring in response to losses in paternity. Previous work on birds and fishes provide mixed empirical evidence for facultative adjustments in male care to losses in paternity. One potential reason for this inconsistency is that males need access to reliable cues of losses in paternity, and that it might be difficult to assess what cues, if any, are used by males. Here we manipulated three cues of losses in paternity in the burying beetle *Nicrophorus vespilloides*: the presence of a (dead) rival male (*N* = 44), the temporary absence of the female (*N* = 41) and the presence of a rival male's cuticular hydrocarbons on the female (*N* = 44). We focused on these three cues because there is evidence that males respond to these cues in other species and there is also evidence that our study species responds to these cues in other contexts. We found no effect of the three cues on the amount of direct or indirect care provided by the male, male weight change, or the number and weight of offspring. Our results provide no evidence that single male parents adjust their investment in the current brood based on cues of losses in paternity. As previous work showed that most wild females arriving on a carcass already store sperm, it is likely that males have evolved a fixed response to female polyandry by mating very frequently with the female.

Keywords:

female promiscuity

life history strategy

paternal care

paternity uncertainty

sperm competition

Parental care can be defined as any parental trait that enhances the fitness of the parent's offspring, and that is likely to have evolved for this function (Smiseth, Kölliker, & Royle, 2012). A major aim in behavioural ecology is to understand why there is so much diversity in the form, level and duration of parental care as well as the extent to which it involves male or female parents (Clutton-Brock, 1991; Royle, Smiseth, & Kölliker, 2012). This diversity is thought to reflect variation in the benefits and costs of parental care to males and females, which depends on factors such as environmental hazards, mating opportunities and relatedness between parents and offspring (Royle et al., 2012). In many species, the relatedness between parents and offspring in a given brood is lower on average for males than for females as a consequence of female promiscuity (Birkhead, 2000). Thus, the evolution of male parental care is expected to be shaped by average losses in paternity due to expected levels of female promiscuity and realized losses in paternity due to variation in actual levels of female promiscuity (Westneat & Sherman, 1993). There is good empirical evidence for evolutionary responses in male involvement in parental care as a consequence of average losses in paternity. For example, in birds, male parental care tends to be lower in species with higher levels of extrapair paternity (Griffith, Owens, & Thuman, 2002).

There is less consistent evidence for facultative adjustments in male care, whereby males reduce the amount of care in response to realized losses in paternity (Alonzo, 2010; Sheldon, 2002; Wright, 1998). One potential explanation for this is that males should adjust their care only if they expect a higher paternity in the future, which may not always be the case (Westneat & Sherman, 1993). Another potential explanation is that such responses are conditional upon the presence of reliable cues that males can use to assess their realized losses in paternity (Alonzo, 2010; Sheldon, 2002; Wright, 1998). The mechanism used by males to assess their paternity losses can be divided into two main categories (Bose, Kou, &

Balshine, 2016; Sherman & Neff, 2003). First, males may respond to indirect cues emanating from the ecological or social environment. Second, males may also respond to direct cues from the offspring's phenotype serving as indicators of genetic relatedness between themselves and the offspring (Hauber & Sherman, 2001). Indirect cues fall into three main categories: (1) the presence of a rival male during the female's fertile period (Neff, 2003), (2) the absence of the female or the male during the female's fertile period (Sheldon, Räsänen, & Dias, 1997) and (3) cues about the female's mating history (Davies, Hatchwell, Robson, & Burke, 1992). Although there is some evidence for all three indirect mechanisms, the relative role of these mechanisms is yet to be investigated in a single system. Furthermore, although the main focus has been adjustments in the amount of male care, males might adjust other behaviours, such as feeding from a resource that they share with the offspring.

Here we investigate responses by caring males to different indirect cues about perceived losses in paternity in the burying beetle *Nicrophorus vespilloides*. This species is an excellent insect model for studying responses by caring males to cues about losses in paternity because (1) there is paternity uncertainty due to female promiscuity (House, Walling, Stamper, & Moore, 2009; House et al., 2008), (2) there is a great amount of variation in the extent to which males contribute towards care for the offspring (Bartlett, 1988; Smiseth & Moore, 2004; Walling, Stamper, Smiseth, & Moore, 2008) and (3) there is evidence that males respond to putative cues about losses in paternity in other contexts (see Methods for further details). This species breeds on carcasses of small vertebrates, which serve as food both for the developing larvae and the parents (Scott, 1998). Multiple males and females will often locate a suitable carcass, leading to intense competition within each sex (Otronen, 1988). Paternity uncertainty occurs due to females storing sperm from previous matings with other males, and females mating with satellite males during the

current breeding attempt (Eggert, 1992; Müller & Eggert, 1989). The resident male copulates frequently with the female, presumably as a means to ensure high paternity (Eggert, 1992; Müller & Eggert, 1989). Either one or both parents may provide extensive care for the offspring before and after hatching (Smiseth & Moore, 2004; Walling et al., 2008). There is no evidence that males use direct cues to access paternity in this species (T. Botterill-James, L. E. Ford, G. M. While, & P. T. Smiseth, personal communication, 26 October 2016). Recent work provides mixed evidence as to whether males adjust their parental care in response to the presence of a male competitor (Benowitz, Head, Williams, Moore, & Royle, 2013; Hopwood, Moore, Tregenza, & Royle, 2015; Luzar, Schweizer, Sakaluk, & Steiger, 2017). However, it is unclear whether the presence of a competitor serves as a cue about lost paternity or as a cue about density and thus the intensity of competition in the near future (Creighton, 2005; Pilakouta, Halford, Rácz, & Smiseth, 2016). Therefore, there is a need for a better understanding of which cues males use as indicators of losses in paternity in this system.

The main aim of this study was to investigate whether males respond to the three indirect cues that are hypothesized to provide males with information about potential losses in paternity. The three cues were (1) the presence of a rival male during the female's fertile period, (2) the absence of the female during the female's fertile period and (3) cues about the female's mating history. In insects, such cues are likely to be based on the cuticular hydrocarbon (CHC) profile of a rival male rubbing off onto the female during contact (Thomas & Simmons, 2009). To control for any effect of our treatments mediated through the female's behaviour, we removed the female at the time of hatching of the offspring. This allowed us to study the male's response to these cues in the absence of any influences on male care due to the female's behaviour. We first investigated effects in terms of amount of

care provided by the male. If males responded to a particular cue, we predicted that males would provide less care when exposed this cue than when exposed to the control treatment. We also investigated effects in terms of the amount of food that the male consumed. If males responded to a particular cue, we predicted that males would provide less care and consume more food (i.e. gain more weight) when exposed this cue than when exposed to the control treatment. Finally, we looked for potential consequences of the experimental treatments for offspring size and number. If males reduced their investment for the brood after being exposed to a cue of lost paternity, we would expect negative consequences for offspring size or survival.

METHODS

Origins of Beetles and Animal Husbandry

All beetles used in this experiment were taken from a stock population of virgin beetles maintained at the University of Edinburgh. We only mated unrelated individuals that had no common ancestors for at least two generations to minimize potential effects of inbreeding (Mattey, Strutt, & Smiseth, 2013). The beetles were housed individually in clear plastic boxes (124 x 82 mm and 22 mm high) containing moist soil and were kept at 21 ± 2 °C under constant lighting (Mattey, Strutt, & Smiseth, 2013). Nonbreeding beetles were fed small pieces of organic beef twice a week.

Experimental Design and Procedures

We selected pairs of unrelated beetles (aged 14–22 days) for use in our three experiments. At the onset of the experiment, the beetles were weighed to the nearest 0.1 mg and then placed in a plastic box (175 x 115 mm and 65 mm high) filled with a thin layer of moist soil and provided with a previously frozen mouse carcass (Livefoods Direct Ltd, Sheffield, U.K.) of standardized size (17–21 g). The beetles were then left for 4 h, allowing them ample time to interact with each other and assess the carcass for breeding. The experimental beetles were then randomly assigned to three experiments, in which we manipulated the potential cues that males might use to assess losses in paternity: (1) the presence of a rival male; (2) the absence of the female; (3) cues about the female's mating history.

In the first experiment, we tested how the focal male parent responded to the presence of a rival male. To this end, we introduced a dead male (killed previously by freezing at -20 °C) to the breeding box for a 2 h period to allow sufficient time for the breeding male to notice its presence. We used a dead male as it avoids introducing any confounding effects on the focal male's behaviour induced by the rival male, such as effects of injuries due to aggressive competition over the carcass or the female (Eggert, Otte, & Müller, 2008). Previous work confirmed that beetles respond to the presence of a dead beetle by using this as a cue to alter their behaviour (Steiger, Whitlow, Peschke, & Müller, 2009). For the control treatment, we introduced a small wooden bead of a similar size and weight as a beetle in the mating box for a corresponding 2 h period. We did this to control for any potential effects of a foreign object being placed in the box.

In the second experiment, we tested how the focal male parent responded to the temporary absence of the female by temporarily removing the breeding females for a 2 h period while the male was left with the carcass. The female was placed temporarily in a box

filled with moist soil (124 x 82 mm and 22 mm high) until she was reunited with the focal male by gently placing her back on the carcass. In the control treatment, the female was briefly picked up and then put back on the carcass at the two time points corresponding to the time when experimental females were moved and placed back on the carcass. We did this to account for any potential effects caused by disturbance when handling females.

In the third experiment, we tested how the focal male parent responded to cues about the female's mating history. We temporarily removed the breeding female from the breeding box and rubbed her against a sexually mature live male for a total of 15 s. We did this to transfer the CHC profile of a rival male onto the breeding female, thereby mimicking any transfer occurring during mating without any mating actually taking place. We did not allow the female to mate with the rival male to avoid any effects due to such matings on the subsequent behaviour of the female. Females were then reunited with the focal male in their breeding box. In the control treatment, we rubbed the females against a wooden bead rather than a rival male to simulate the similar level of disturbance as for the experimental females, but without transferring any CHCs.

Once we had conducted the experimental treatments as described above, we monitored subsequent effects on male parental care, male weight change and the male's breeding success. Previous work on this species suggests that parental care and or weight change may be conditional upon the age and size of the brood and the female's behaviour (Pilakouta, Richardson, & Smiseth, 2016; Smiseth, Darwell, & Moore, 2003; Smiseth & Moore, 2004). Thus, to exclude any confounding effects on male care due these factors, we removed the female and provided males with a standardized brood based on established protocols (Pilakouta et al., 2015; Smiseth, Lennox, & Moore, 2007). Therefore, we left the pairs until the female had completed egg laying. We then removed the female before the larvae were

expected to hatch. At this time, we also transferred the male and the mouse carcass to a new breeding box to separate them from the eggs. The eggs were left to hatch in the original box. Once larvae had started to hatch, they were used to generate experimental broods composed of 15 newly hatched larvae (*N* = 134 broods). Experimental broods were always composed of larvae of mixed maternity and derived from all three experimental treatments to control for any potential effects of the treatments on larval behaviour or development. Previous work has shown that parents do not discriminate between own and foster larvae, but they have a temporal form of kin discrimination whereby they kill any larvae hatching earlier than their own (Müller & Eggert, 1990). Thus, to avoid infanticide, we only provided males with a standardized brood once their own brood had started hatching.

We conducted behavioural observations 24 h (±30 min) after males had been provided with an experimental brood, which corresponds to the peak of parental food provisioning in this species (Smiseth et al., 2003; Smiseth et al., 2007). We used instantaneous sampling, scoring males' parental behaviours every 1 min for 30 min in accordance with established protocols (Smiseth & Moore, 2002). We counted the number of scans during which each male parent was providing 'direct care', defined as when the male was provisioning food to the larvae (i.e. mouth-to-mouth contact with larvae), or manipulating or regurgitating carrion within the crater (the opening on top of the carcass), and 'indirect care', defined as when the male was manipulating the surface of the carcass (excluding the crater) to remove fungus and bacteria, excavating the crypt (depression in the soil within which the carcass was buried), or moving the carcass within the crypt (Jenkins, Morris, & Blackman, 2000; Walling et al., 2008).

Once observations were carried out, we left the male undisturbed until larval dispersal from the carcass. Larval dispersal was defined to occur when at least two larvae

had moved away from the carcass and into the surrounding soil. At this stage, we counted and weighed the larvae to provide information on the male's breeding success. We also weighed the male to obtain information on male weight change relative to his weight at the onset of the experiment. Only these broods that reached dispersal (*N* = 129 out of 134) were included in our analyses. The final sample sizes were as follows: *N* = 44 for experiment 1 (25 experimental broods and 19 control broods), *N* = 44 for experiment 2 (23 experimental broods and 21 control broods) and *N* = 41 for experiment 3 (21 experimental broods and 20 control broods). We were not able to obtain behavioural data for six of these broods because of technical issues, and these broods were therefore excluded from the analyses of direct and indirect care. To minimize observer bias, blinded methods were used when all data were recorded and/or analysed.

Statistical Analyses

All analyses were performed using R version 3.0.1 (R Core Team, 2013). For each of the three experiments, we tested for effects of the treatments (treated as a binary factor: experimental treatment or its respective control) by including it as the only explanatory factor. In models where direct care and indirect care were the response variables, we used zero-inflated linear models ('glmmadmb', package 'glmmADMB' for negative binomial distributions) because of the high proportion of males that did not provide either direct or indirect care during the 30 min observation period. In models on male weight at dispersal, we used linear models ('lm', package 'stats') with both treatment and initial male weight as dependent variables and male weight at dispersal as the response variable. This analysis allowed us to test for an effect of the experimental treatments on male weight at dispersal,

while controlling for effects due to variation in the initial weight of the male. To test for effects of the experimental treatments on average larval weight and brood size at dispersal, we used linear models with treatment as the only explanatory factor.

Ethical Note

All procedures performed were in accordance with the legal requirements of the U.K., as well as all institutional standards of The University of Edinburgh.

RESULTS

There was no evidence that males adjusted their levels of care in response to any of the three potential cues about losses in paternity (Fig. 1). Males did not reduce the amount of time spent on direct care in response to the temporary presence of a dead male (likelihood ratio test, LR: $\chi^2 = 2.08$, $P = 0.15$) or the temporary removal of the female (LR: $\chi^2 = 1.51$, $P = 0.15$). Although males did not reduce the amount of time spent on direct care in response to cues about the female's mating history when the female had been rubbed against a rival male (LR: $\chi^2 = 3.52$, $P = 0.06$), there was a nonsignificant tendency for males to decrease care when their mate had been rubbed with cues of a rival male (Fig. 1). Furthermore, males did not reduce the amount of time spent on indirect care in response to the presence of a dead male (LR: $\chi^2 = 0.002$, $P = 0.96$), the temporary removal of the female (LR: $\chi^2 = 1.77$, $P = 0.18$), or cues about the female's mating history when the female had been rubbed against a rival male (LR: $\chi^2 = 1.30$, $P = 0.25$).

There was no evidence that male weight change was influenced by the three potential cues about losses in paternity (Fig. 2). As expected, male weight at dispersal was strongly influenced by the male's initial weight (all P values <4 e^{-11}). However, male weight at dispersal was not affected by the temporary presence of a dead male ($F_{1,41}$ = 0.08, P = 0.77), the temporary removal of the female ($F_{1,41}$ = 0.55, P = 0.46) or cues about the female's mating history ($F_{1,38}$ = 0.18, P = 0.68; Fig. 2).

Finally, there was no evidence that the male's breeding success was influenced by the three potential cues about losses in paternity (Fig. 3). Neither brood size nor average larval weight at dispersal was affected by the temporary presence of a dead male (brood size: $F_{1,42}$ = 0.95, P = 0.33; average larval weight: $F_{1,42}$ = 1.16, P = 0.29), the temporary removal of the female (brood size: $F_{1,42}$ = 0.53, P = 0.47; average larval weight: $F_{1,42}$ = 0.06, P = 0.81) or cues about the female's mating history (brood size: $F_{1,39}$ = 0.14, P = 0.71; average larval weight: $F_{1,42}$ = 0.08, P = 0.78).

DISCUSSION

We found no evidence that single *N. vespilloides* males reduced their amount of care or increased the amount of food they consumed in response to three indirect cues about losses in paternity. Our results derive from an experimental design in which we focused on three indirect cues that have been shown to indicate a loss in paternity in previous work on other species: the presence of a rival male, the absence of the female and cues about the female's mating history (Davies et al., 1992). We note that we manipulated the presence of a rival male by presenting a dead rival, which means that our design removes any potential confounding effects due to the rival's behaviour, such as injury to the focal male caused by

aggressive interactions with the rival. Furthermore, we focused on several forms of male parental care, including direct and indirect care, and we also measured male weight change, indicative of a potential shift towards investment in self-maintenance. Finally, we measured the fitness consequences of the variation in the male's behaviour in response to losses in paternity. Therefore, we conclude that our results suggest that single *N. vespilloides* males do not adjust their parental behaviour in response to cues about lost paternity. Below we provide a more detailed discussion of our results and their implications for our understanding of male responses to cues about lost paternity.

There are three potential explanations for why males did not adjust their behaviour in response to cues about lost paternity. First, males may lack the ability to perceive any of the three cues. This explanation seems highly unlikely as previous work on this species has shown that males respond to the presence of a dead beetle (Steiger, Peschke, & Müller, 2008) and their partner's CHC profile (Steiger, Franz, Eggert, & Müller, 2008) in other contexts. Moreover, there is also evidence that males respond to the absence of their partner and that males do so after an absence of 45 min (Steiger & Müller, 2010). We removed the female for 2 h in the present study, thus giving males ample time to notice that their partner was absent. Second, males may perceive the cues but respond in a manner that we failed to detect, such as by adjusting the amount of prehatching care they provide. While we cannot completely rule out this possibility, it seems unlikely that males adjusted a behaviour that affected either their own or their offspring fitness as there were no differences between experimental and control males in weight change, brood size or mean larval mass. Third, although males may perceive cues about losses in paternity, they may choose not to respond to them. Our results are consistent with this explanation given that

we found no evidence that the three putative cues about lost paternity affected either male behaviour or male and offspring fitness traits.

Two previous studies on this species have suggested that males adjust their parental behaviour in response to the presence of a male competitor (Benowitz et al., 2013; Hopwood et al., 2015). Benowitz et al. (2013) found that young males provided less prenatal care whereas old males provided more prenatal care when they were exposed to a male competitor. Hopwood et al. (2015) found that males cared for their offspring longer when the males had been exposed to a male competitor. Although males might increase their care in response to losses in paternity, this pattern is rare and has only been reported when paternity losses covary with brood size (Alonzo & Heckman, 2010). In contrast, a very recent study found that mating opportunities had no effect on a subordinate male's decision to provide care (Luzar et al., 2017), thus concurring with the results of our study. One potential explanation for this discrepancy is that males only respond to losses in paternity in the presence of the female. While we cannot rule out this possibility, we note that the presence of a male competitor might serve as a cue about other things than lost paternity such as population density and intensity of competition for resources in the near future (Creighton, 2005; Pilakouta, Halford et al., 2016). Indeed, this explanation is consistent with the finding that males also provide more care when exposed to the presence of a female competitor (Hopwood et al., 2015), which seems unlikely to be a response by the male to losses in paternity. Furthermore, previous work has shown that females also respond to the presence of a same-sex competitor by increasing the amount of care they provide (Creighton, 2005; Pilakouta, Halford et al., 2016). Thus, in light of our results and previous work on the same system, we conclude that there is inconsistent evidence for males adjusting their parental behaviour to cues about lost paternity and we encourage experimental work that compares

male responses to cues about losses in paternity under uniparental and biparental conditions.

There are several reasons for why it might be adaptive for *N. vespilloides* males to ignore cues about lost paternity. First, previous work has shown that 93% of sexually mature females arriving on a carcass in the wild have previously mated with another male (Müller & Eggert, 1989). Thus, males may be under selection to assume that females always store sperm from previous matings with other males. Nevertheless, the resident males fertilize 92% of the female's eggs (range 75–100%), and males seem to achieve such a high paternity by mating very frequently with the female (on average 103 times in 48 h; *N*=8, Müller & Eggert, 1989). Thus, *N. vespilloides* males might have evolved a fixed response to the high risk of sperm competition in this system, which involves high frequency of mating with the female. Second, it would only be beneficial for males to reduce the amount of care they provide to their current brood in response to lost paternity if they can expect higher paternity in future breeding attempts (Westneat & Sherman, 1993). It is unclear whether this condition is met in our study species, given that *N. vespilloides* breeds on carcasses of small vertebrates, a rich but scarce resource (Scott, 1998). Thus, future breeding opportunities are likely to be severely limited, in which case it would be risky to save resources for future reproduction. Nevertheless, a recent study found that males that had cared for a brood produced higher quantities of sex pheromones and attracted three times more females than males that had not raised a brood (Chemnitz, Bagrii, Ayasse, & Steiger, in press). These results suggest that caring males might be more successful at attracting females in future breeding attempts, although it is unclear whether they also have higher paternity in future breeding attempts.

Until now, most studies on male responses to cues about losses in paternity have been conducted on birds and fishes (Mank, Promislow, & Avise, 2005; Wright, 1998). The reason for this is simply that biparental care and uniparental male care are relatively common in these taxa compared to many other taxa (Balshine, 2012). These studies have provided mixed results (Sheldon, 2002). For example, there is evidence that males reduce the amount of care they provide in response to the presence of rival males in bluegill sunfish, *Lepomis macrochirus* (Neff, 2003), the take-over of another male's nest in the plainfin midshipman fish, *Porichthys notatus* (Bose et al., 2016) and the temporary removal of the female in collared flycatchers, *Ficedula albicollis* (Kempenaers & Sheldon, 1996; Sheldon et al., 1997). Meanwhile, other studies found no evidence of a response by males to the same cues in eastern bluebirds, *Sialia sialis*, and tree swallows, *Tachycineta bicolor* (Kempenaers, Lanctot, & Robertson, 1998; MacDougall-Shackleton & Robertson, 1998; Whittingham, Dunn, & Robertson, 1993). We suggest that it is now timely to expand beyond the current focus on birds and fishes. Although male care is relatively uncommon in several other taxa (Ridley, 1978), it has evolved repeatedly in some amphibians (Balshine, 2012) as well as in arthropods and other invertebrates (Trumbo, 2012). For example, biparental care occurs in burying beetles, dung beetles and passalid beetles, while uniparental male care occurs in giant waterbugs and assassin bugs (Choe & Crespi, 1997). Currently, we lack information on paternity in many of these species. However, a recent study found high levels of extrapair paternity in a passalid beetle with biparental care (Dillard, 2017). Thus, there is a need for further work on the levels of extrapair paternity and male responses to lost paternity in arthropods with biparental care or uniparental care.

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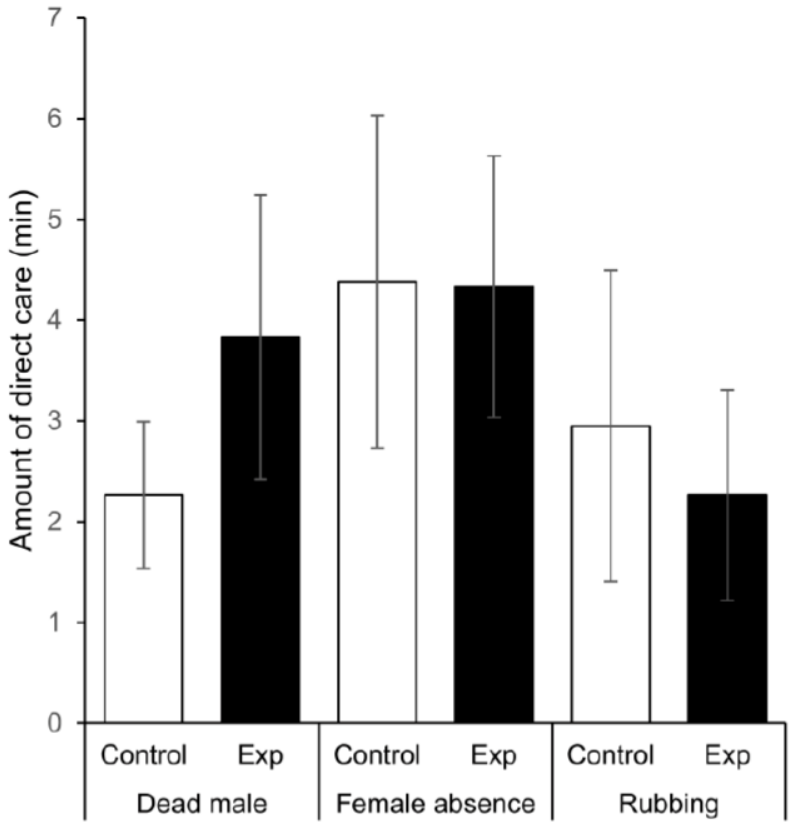
Figure 1. Mean ±SE time that males spent providing direct care during 30 min behavioural observations after being exposed to three different cues of paternity loss and their respective controls.

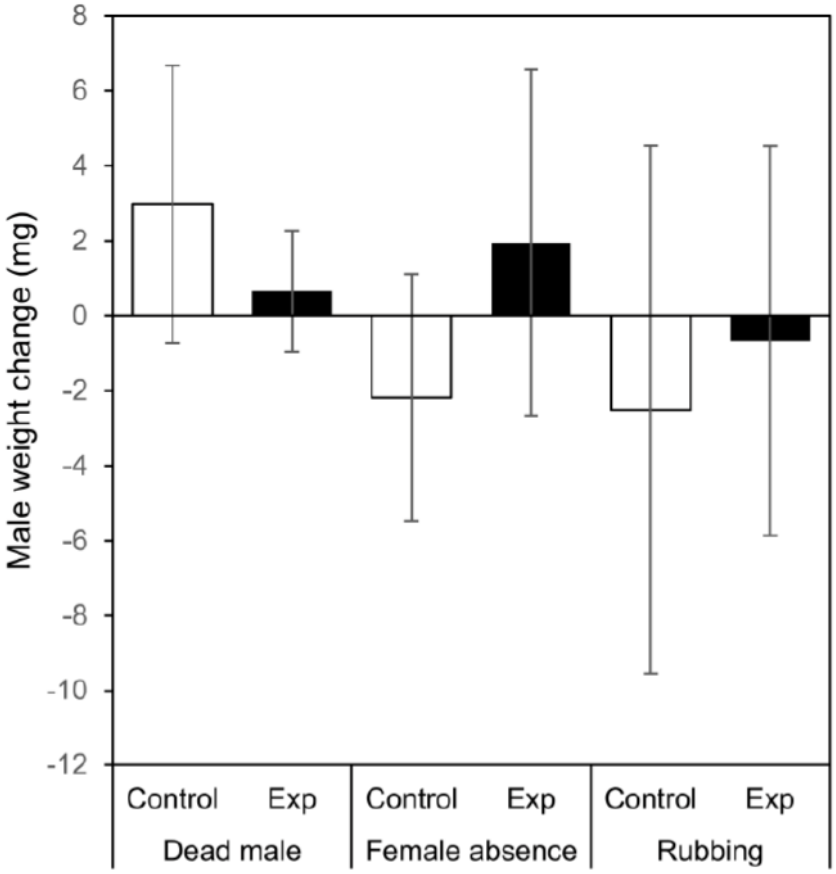
Figure 2. Mean ±SE male weight change from mating to dispersal after being exposed to the three tested cues of paternity loss and their respective controls.

Figure 3. Mean ±SE number of larvae reaching dispersal for the three experimental treatments and their respective controls.

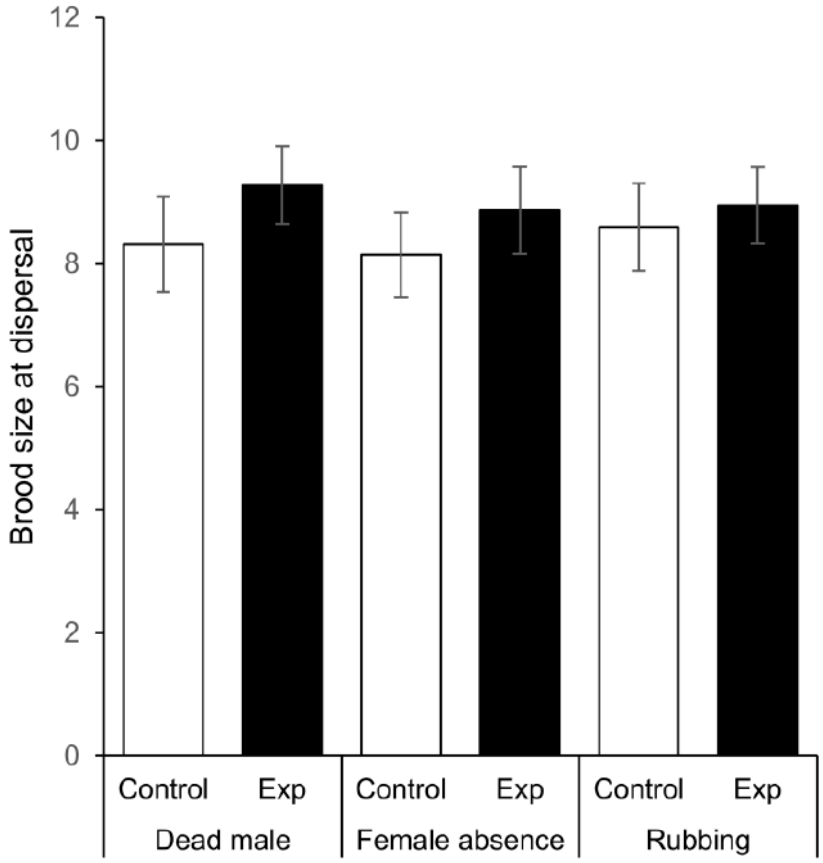
[Figure 1]


Amount of direct care (min)
0
1
2
3
4
5
6
7
Control
Exp
Dead male
Control
Exp
Female absence
Control
Exp
Rubbing

[Figure 2]


Male weight change (mg)
8
6
4
2
0
-2
-4
-6
-8
-10
-12
Control
Exp
Control
Exp
Control
Exp
Dead male
Female absence
Rubbing

[Figure 3]


Brood size at dispersal
12
10
8
6
4
2
0
Control
Exp
Control
Exp
Control
Exp
Dead male
Female absence
Rubbing